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Original article

Comparative evaluation of *Amblyomma ovale* ticks infected and noninfected by *Rickettsia* sp. strain Atlantic rainforest, the agent of an emerging rickettsiosis in Brazil

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ABSTRACT

In 2010, a novel spotted fever group rickettsiosis was reported in the Atlantic rainforest coast of Brazil. The etiological agent was identified as *Rickettsia* sp. strain Atlantic rainforest, and the tick *Amblyomma ovale* was incriminated as the presumed vector. The present study evaluated under laboratory conditions four colonies of *A. ovale*: two started from engorged females that were naturally infected by *Rickettsia* sp. strain Atlantic rainforest (designated as infected groups); the two others started from noninfected females (designated as control groups). All colonies were reared in parallel from F_0 engorged female to F_2 unfed nymphs. Tick-naïve vesper mice (*Calomys callosus*) or domestic rabbits were used for feeding of each tick stage. *Rickettsia* sp. strain Atlantic rainforest was preserved by transstadial maintenance and transovarial transmission in *A. ovale* ticks for at least 2 generations (from F_0 females to F_2 nymphs), because nearly 100% of the tested larvae, nymphs, and adults from the infected groups were shown by PCR to contain rickettsial DNA. All vesper mice and rabbits infested by larvae and nymphs, and 50% of the rabbits infested by adults from the infected groups seroconverted, indicating that these tick stages were vector competent for *Rickettsia* sp. strain Atlantic rainforest. Expressive differences in mortality rates and reproductive performance were observed between engorged females from the infected and control groups, as indicated by 75.0% and 97.1% oviposition success, respectively, and significantly lower egg mass weight, conversion efficiency index, and percentage of egg hatching for the infected groups. Our results indicate that *A. ovale* can act as a natural reservoir for *Rickettsia* sp. strain Atlantic rainforest. However, due to deleterious effect caused by this rickettsial agent on engorged females, amplifier vertebrate hosts might be necessary for persistent perpetuation of *Rickettsia* sp. strain Atlantic rainforest in *A. ovale* under natural conditions.

1. Introduction

In 2010, a novel spotted fever group (SFG) rickettsiosis was reported in the Atlantic rainforest coast of Brazil, based on a patient presenting an acute disease characterized by fever, muscle and joint pain, rash, and an inoculation eschar at the tick bite site (Spolidorio et al., 2010). The etiological agent was identified as *Rickettsia* sp. strain Atlantic rainforest, which was shown to be genetically closest to the tick-borne pathogens *Rickettsia africae*, *Rickettsia parkeri*, and *Rickettsia sibirica* (Spolidorio et al., 2010). A subsequent epidemiological study at the site where the above patient acquired the infected tick revealed *Amblyomma ovale* as the predominating tick species, which was found under a ≈10% infection rate by *Rickettsia* sp. strain Atlantic rainforest (Szabó et al., 2013). Because *A. ovale* is one of the most common human-biting ticks in South America (Guglielmone et al., 2006), it was suggested that this tick species is a natural vector *Rickettsia* sp. strain Atlantic rainforest to humans (Szabó et al., 2013). Other field studies in Atlantic rainforest areas of Brazil also reported ≈10% infection rate by *Rickettsia* sp. strain Atlantic rainforest in *A. ovale* ticks, in addition to other tick species such as *A. aureolatum* and *Rhipicephalus sanguineus* sensu lato (Sabatini et al., 2010; Medeiros et al., 2011; Barbieri et al., 2014).

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A second human clinical case of SFG rickettsiosis, caused by *Rickettsia* sp. strain Atlantic rainforest (referred as 'strain Bahia'), was reported in another Atlantic rainforest area of Brazil (Silva et al., 2011). More recently, this agent was reported infecting *A. ovale* ticks in Colombia (Londoño et al., 2014), Belize (Lopes et al., 2016), and in the Pantanal biome of Brazil (Melo et al., 2015), indicating that this pathogen might have a wide distribution in Latin America, primarily associated with *A. ovale* ticks.

A. ovale has a broad distribution in the Neotropical region, with established populations from Mexico to Argentina (Guglielmone et al., 2003). The adult stage of *A. ovale* is a typical parasite of Carnivora hosts, including domestic dogs, although tapirs and peccaries are also commonly reported in more preserved areas (Aragão, 1936; Labruna et al., 2005, 2010). Differently from the adult stage, immature stages (larvae and nymphs) of *A. ovale* feed primarily on small rodents (Jones et al., 1972; Guglielmone et al., 2003; Saraiva et al., 2012; Szabó et al., 2013; Martins et al., 2015; Sponchiado et al., 2015). However, there have some records on passerine birds (Ogrzewalska et al., 2009; Luz et al., 2012; Pacheco et al., 2012; Ramos et al., 2015), suggesting a possible role of birds in the dispersion of *A. ovale* over large distances.

Because our knowledge on the ecology of *Rickettsia* sp. strain Atlantic rainforest is still very incipient, the present study evaluated for the first time (i) the occurrence of transovarial transmission (passage of viable rickettsiae from tick female to its offspring) and transstadial transmission (maintenance of the infection during tick ontogeny) of the agent in *A. ovale* ticks, (ii) if the infection with this rickettsial agent causes any deleterious effect on its tick host, and (iii) the vector competence of all feeding stages of *A. ovale*.

2. Material and methods

2.1. Formation of tick colonies

In February 2011, approximately 50 *A. ovale* adult ticks were collected from naturally infested dogs in Barra do Una, Peruíbe municipality, state of São Paulo, Brazil (24°25′ S; 47°03′ W), during an epidemiological study of *Rickettsia* sp. strain Atlantic rainforest (Szabó et al., 2013). Barra do Una is the locality of the index human case of *Rickettsia* sp. strain Atlantic rainforest-caused spotted fever (Spolidorio et al., 2010). Ticks were brought alive to the laboratory, where female engorgement was completed by feeding on a tick-naïve uninfected New Zealand white rabbit (*Oryctolagus cuniculus*). A total of 32 engorged females were allowed to oviposit inside an incubator adjusted to 25 °C and 90% relative humidity. Small samples of eggs (1 pool of ≈50 eggs per female) and the subsequent hatched larvae (1 pool of ≈20 larvae per female) derived from each engorged female were subjected to DNA extraction, and subsequently tested by a real time-PCR assay targeting a 147-bp fragment of the rickettsial *gltA* gene (protocols mentioned below). Positive samples by real time-PCR were subsequently tested by a conventional PCR using primers Rr190.70p and Rr190.602n, targeting a 532-bp fragment of the rickettsial 190-kDa outer membrane protein gene (*ompA*), as previously described (Regnery et al., 1991). Egg and larval samples from 2/32 (6.3%) engorged females yielded amplicons by both PCR protocols. The *ompA* PCR products were sequenced (protocol mentioned below), resulting in *ompA* fragments 100% identical to corresponding sequences of *Rickettsia* sp. strain Atlantic rainforest in GenBank (GQ855237). Based on these results, the larval offspring (F_1) from these two infected females were used to start the two *Rickettsia*-infected groups of the present study, designated as I1 and I2. At the same time, the larval offspring (F_1) from two PCR-negative uninfected females were separated to start the two control groups, designated as C1 and C2.

2.2. Tick infestations

The F_1 unfed larvae of infected and control groups were reared separately in the laboratory until the next generation, when ticks were F_2 unfed nymphs. Throughout the experiment, infestations with infected and control groups were done in parallel; all infested animals were held in the same room under the same environmental conditions. Laboratory-reared vesper mice (*Calomys callosus*) were infested with larvae (2–3 mice/larval generation/experimental group), whereas New Zealand white rabbits were infested with nymphs or adult ticks (2 rabbits/tick stage/experimental group). All animals used in the present study were obtained from an animal room with no history of tick infestation or tick-borne disease or any contact with acaricides or antibiotic drugs.

Larval and nymphal infestations consisted of ≈500 and 60 ticks, respectively, per host, whereas adult infestations consisted of 10 male and female pairs per rabbit. Larval infestations of *C. callosus* consisted of releasing the ticks on the dorsum of the animals, as previously described (Martins et al., 2012). Infestation of each rabbit was performed inside a feeding chamber glued to its shaved dorsum, as previously described (Pinter et al., 2002). All infested rabbits had their temperature rectally measured daily from the day of infestation (day 0) to 21 days postinfestation. Rabbits were considered febrile if rectal temperatures were >40.0 °C (Monteiro, 1933). Because of its small size, rectal temperature of *C. callosus* could not be performed. Moreover, attempts to measure its temperature by using an infrared thermometer showed to be inefficient; thus, body temperature of *C. callosus* could not be gathered throughout the study.

Naturally detached engorged larvae, nymphs, or female ticks were recovered daily from each infested animal of both groups, as previously described (Martins et al., 2012). Recovered engorged ticks were immediately taken to a single incubator adjusted to 25 °C and 95% relative humidity for molting (for engorged larvae and nymphs) or for egg laying and incubation (for engorged females). From each experimental group (I1, I2, C1, C2), 100 engorged larvae or 50 engorged nymphs were chosen for evaluation of molting success (the proportion of engorged ticks that successfully complete ecdysis). Engorged females had their individual weight measured the day they detached from the host. In addition, the total egg mass deposited by each female was weighed on the day of the end of oviposition and a conversion efficiency index (CEI = mg egg mass/mg engorged female × 100), which measures the efficiency with which a female tick converts body weight into eggs (Bennett, 1974), was determined for each female that oviposited. Percentage of egg hatching for each egg mass was visually estimated (Labruna et al., 2000).

This study has been approved by the Ethics Committee for the Use of Animals (CEUA) of the Faculty of Veterinary Medicine of the University of São Paulo (protocol number 2191/2011).

2.3. Molecular analyses

During the study, samples of F_1 unfed nymphs (30 individuals) and adults (20), and F_2 eggs (10), unfed larvae (10) and nymphs (20) from each of the experimental groups (I1, I2, C1, C2) were submitted for DNA extraction by using guanidine thiocyanate, as described previously (Sangioni et al., 2005), and tested individually by real-time PCR assay using primers CS-5 (Guedes et al., 2005) and CS-6, which amplify a 147-bp fragment of the rickettsial *gltA* gene. These primers have shown sensitivity down to a single copy of *R. rickettsii* (Labruna et al., 2004). A fluorogenic probe (5′ 6-FAM d, BHQ-1 3′) (Integrated DNA Technologies, San Diego, CA) positioned 76 bp downstream of the forward primer and 3-bp upstream of the reverse primer was used in the reactions (Labruna et al., 2004). Reactions were performed in a 7500 Real Time PCR Systems